Original Article

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Aberrant expression of Notch3 predicts poor survival for hepatocellular carcinomas

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Summary **Using a meta-analysis on gene expression data of hepatocellular carcinomas (HCC) from the Oncomine database, we identified that the Notch3 gene ranked as the highest up-regulated Notch pathway member in HCC tissues compared with normal liver tissues. We further detected the expression of Notch3 in 95 cases of HCC samples by immunohistochemistry, and evaluated its clinicopathological and prognostic significance. We confirmed that Notch3 is overexpressed in HCC tissues compared with normal liver tissues, and a high expression level of Notch3 was significantly associated with bigger tumor size ($p = 0.014$), multiple tumors ($p = 0.026$), late tumor-node-metastasis (TNM) stage ($p < 0.01$) and shorter overall survival ($p = 0.002$). Furthermore, high Notch3 expression emerged as a significant independent prognostic factor in multivariate analysis. In conclusion, we identified a positive association between Notch3 expression with more aggressive traits and shorter survival in HCC. Our findings suggest that Notch3 might be an important regulator in the development of HCC, and a potential therapeutic target for patients with HCC.**

Keywords: Notch pathway, Notch3, hepatocellular carcinomas, immunohistochemistry, prognosis

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth or sixth most prevalent cancer worldwide, including China (*1*). HCC has a relatively aggressive clinical course, even when receiving intensive treatment including curative resection, percutaneous ablation therapy, chemotherapy or liver transplantation, advanced HCC patients still have a poor prognosis. Therefore, there is an urgent need to develop novel prognostic biomarkers and therapeutic targets for HCC to improve its clinical outcome.

The Notch signaling pathway is an evolutionarily highly conserved mechanism involved in cell fate determination during development (*2*). Aberrant Notch signaling activiation has been highlighted as having a potential role in cancer stem cells (*3*). High Notch pathway activity correlates with more aggressive or treatment-resistant phenotypes, and poor prognosis in multiple cancers (*4*).

In this present study, we performed a meta-analysis on the gene expression datasets of HCC from the Oncomine database, and identified Notch3 as ranked at the top of significantly overexpressed Notch pathway genes in HCC. Several previous studies investigated the expression of Notch3 in HCC, however, the results were inconsistent (*5,6*). Especially, the clinicopathological and prognostic significance of Notch3 in HCC has seldom been investigated. Therefore, in this study, we examined the expression of Notch3 protein in human HCC tissues and adjacent normal liver tissues by immunohistochemistry, to clarify the possible roles of Notch3 in the malignant progress and prognosis of HCC.

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2. Materials and Methods

2.1. *Data mining*

Oncomine cancer microarray database (*https://www.oncomine.org*) was used to mine the mRNA expression profile data of HCC tissue relative to normal liver tissue according to the methodology established by Oncomine (*7*). Six publicly available datasets of gene expression profiles (Chen Liver, Mas Liver, Roessler Liver, Roessler Liver 2, TCGA Liver and Wurmbach Liver) were selected for the mata-analysis. All the genes, in which mRNA levels increased by more than two fold in HCC tissue vs. normal liver tissue were selected. The gene ranks across 6 datasets were compared, and then a concept filter *Notch signaling pathway - KEGG Pathway* was used to identify known Notch signaling pathway memebers overexpressed in HCC at the mRNA level.

2.2. *Patients*

Archived formalin-fixed paraffin-embedded HCC specimens undergoing hepatectomy were obtained between January 2005 and January 2009. The diagnoses of HCC patients were confirmed by histopathology. All the HCC patients received curative liver resection which was defined previously (*8*). Patients with confirmed recurrence received further treatment including resection, transarterial chemoembolization (TACE) or radiaofrequency ablation (RFA). None of the patients received neoadjuvant chemotherapies, radiation therapies or immunotherapies. Overall survival (OS) was calculated from the date of surgery until the date of death or last follow-up. Ethics approval to start this study was obtained from the local human research ethics committees. The clinicopathologic variables, such as gender, age, tumor size, histological grade, tumor-node-metastasis (TNM) stage, status of cirrhosis, tumor numbers, hepatitis status, capsular formation and vein invasion are seen in Table 1.

2.3. *Immunohistochemistry*

A total of 95 archived formalin-fixed paraffin-embedded HCC specimen tissues and 20 cases of adjacent normal liver tissue were included in the immunohistochemical study. Immunostaining was performed in accordance with the standard streptavidin-peroxsidase (SP) procedures according to the manufacturer's protocol (Zymed Laboratories, South San Francisco, CA, USA). Antigen retrieval was performed using microwave heating in sodium citrate buffer. Immunostaining was performed with a rabbit polyclonal antibody against Notch3 (AbCam Biotechnology, Abcam China, Hong Kong). Immunostaining was visualized with 3,3'-Diaminobenzidine (DAB) substrate, and counterstained with hematoxylin. Non-specific rabbit IgG was used as a negative control. The immunohistochemical results of Notch3 expression were assessed according to the criteria described previously (*9,10*). Because Notch3 staining was almost homogeneous within each tumor, Notch3 immunostaining intensity was used to evaluate immunohistochemical results and classified into three expression levels: none (no staining even at × 400 field), low (weak staining, staining obvious only at × 400 field), and high (moderate or strong staining, staining obvious at ×100 or ×40 field).

2.4. *Statistics*

The relationship between Notch3 and clinicopathological characteristics among different groups was compared using the χ^2 test or *t*-test when appropriate. Overall survival curves were made using the Kalpan-Meier method and evaluated using the Log-Rank test. Multivariate analysis of clinicopathological variables and Notch3 expression was performed using the Cox proportional hazard regression model. Statistical analyses were completed using SPSS 15.0 and a $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Data mining on Oncomine database*

We systematically compared mRNA gene expression levels between HCC and normal liver tissuefrom 6 datasets from the Oncomine database, then the up-regulated genes in HCC tissues belonging to Notch pathway members were further filtered out. The top 20 up-regulated Notch pathway memebers in HCC are listed in Figure 1, among which Notch3 was the highest ranked gene (median rank = 674) with the most significant *P* value of 3.20×10^{-6}. In a previous study, Gramantieri *et al*. (*5*) also indicated that Notch3 mRNA was abnormally accumulated in a major proportion of HCC tumors, but was negative in normal liver and and cirrhotic tissue. These findings support the hypothesis of an activation of Notch signalling by Notch3 in HCC. Therefore, we selected Notch3 for further validation in a cohort of HCC samples by immunohistochemistry.

3.2. *Immunohistochemical results of Notch3 in HCC and normal liver tissue*

Our immunohistochemical results indicated that Notch3 expression level was significantly higher than that in normal liver tissue, which is consistent with the finding in the meta-analysis on datasets from the Oncomine database. In all the HCC samples, 70 cases (73.7%) indicated Notch3 positive staining, including 44 cases (46.3%) with low expression and 26 cases (27.4%) with high expression according to the staining evaluation criteria we established, whereas only 5 cases of normal

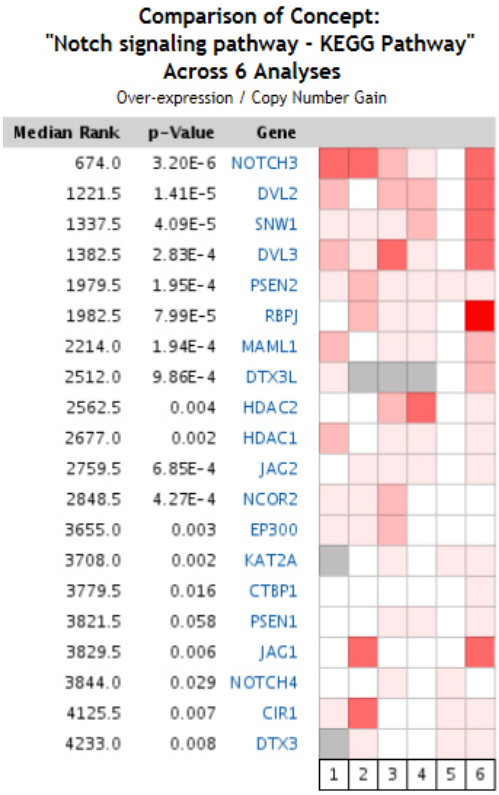

Figure 1. Oncomine heat map of up-regulated genes in clinical HCC samples compared to normal liver tissue filtered by Notch3 pathway concept. Notch3 ranked at the top of up-regulated Notch pathway members.

liver samples demonstrate even low Notch3 expression. Positive staining of Notch3 was located in the cytoplasm and nucleus of cancer cells. Representative immunostaining images are seen in Figure 2.

3.3. *Association between Notch3 expression and clinicopathological features*

The association between Notch3 expression and clinicopathological features of HCC are shown in Table 1. We found that Notch3 expression in HCC was significantly associated with tumor size (p = 0.014), TNM stage (p <0.01) and tumor numbers (p = 0.026). High Notch3 expression was more frequently seen in bigger, multiple or later stage HCC tumors. No significant association was obeserved between Notch3 expression and other clinicopathological traits.

3.4. *The prognostic significance of Notch3 for HCC patients*

Survival curves were plotted using the Kaplan-Meier method (Figure 3). The results indicated that HCC tumors with high Notch3 expression had a significantly shorter survival time, compared with those with no or low expression (hazards ratio = 1.858, 95% confidence interval = 1.264-2.731, p = 0.002). All of the HCC

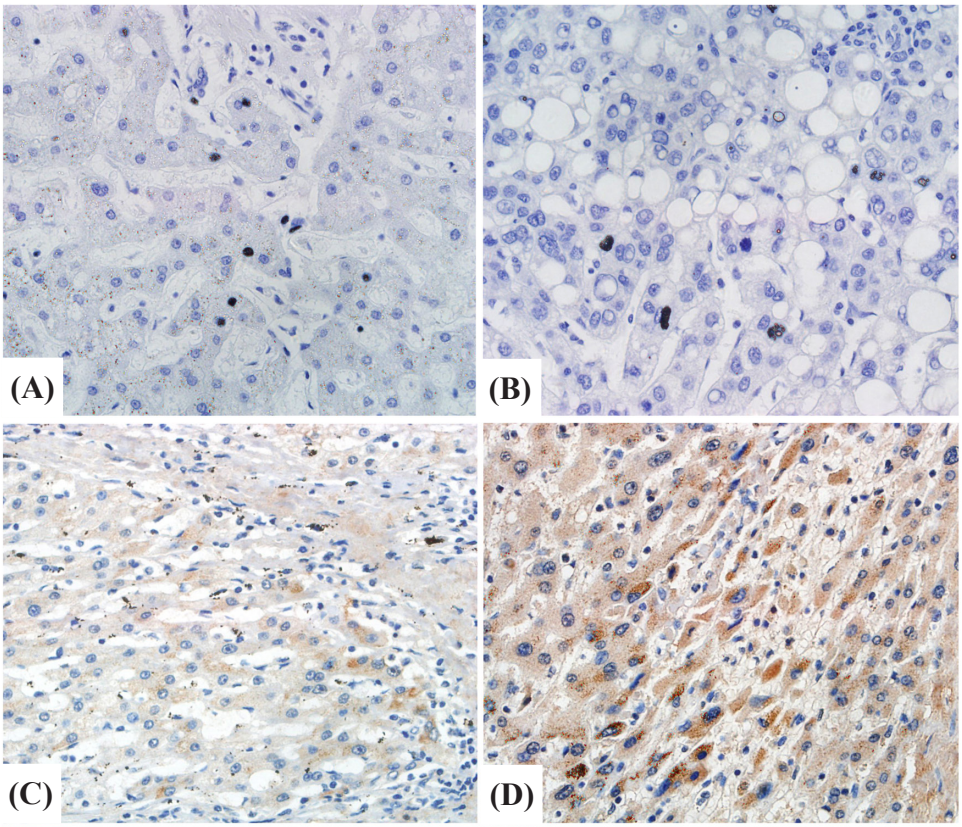

Figure 2. Representative images of Notch3 immunostaining in this study. (A) no Notch3 immunostaining in normal liver tissues; **(B)** no Notch3 immunostaining in HCC tissues; **(C)** low expression of Notch3 in HCC tissues; **(D)** high expression of Notch3 in HCC tissues.

Table 1. Association between Notch3 expression and clinicopathological characteristics in HCC samples

Variables (*n*)	Notch3 expression level			*p* value
	None (%)	Low (%)	High (%)	
Age (year)	54.0 ± 9.1	53.7 ± 8.2	55.5 ± 10.2	0.328
Gender				
Male (78)	20 (25.6)	37 (47.4)	21 (26.9)	0.894
Female (17)	5 (29.4)	7 (41.2)	5 (29.4)	
Tumor size				
T1-2 (74)	21 (28.4)	38 (51.5)	15 (20.3)	0.014
T3 (21)	4 (19.1)	6 (28.6)	11 (52.4)	
Histological grade				
1 (12)	3 (25.0)	6 (50. 0)	3 (25.0)	
2 (60)	13 (21.7)	29 (48.3)	18 (30.0)	0.445
3 (21)	9 (42.9)	7 (33.3)	5 (23.8)	
TNM Stage				
I (58)	18 (31.0)	33 (56.9)	7 (12.1)	< 0.001
II (15)	1 (6.7)	6 (40.0)	8 (53.3)	
III (19)	6 (31.6)	5 (26.3)	8 (42.1)	
IV (3)	0 (0)	0 (0)	3 (100)	
Cirrhosis				
Presence (34)	8 (23.5)	18 (52.9)	8 (23.5)	0.624
Absence (61)	17 (27.9)	26 (42.6)	18 (29.5)	
Tumor nodules				
Single (81)	23 (28.4)	40 (49.4)	18 (22.2)	0.026
Multiple (14)	2 (14.3)	4 (28.6)	8 (57.1)	
Hepatitis				
Negative (32)	5 (15.6)	16 (50.0)	11 (34.4)	0.212
HBV/HCV (63)	20 (31.7)	28 (44.4)	15 (23.8)	
Capsular formation				
Presence (44)	13 (29.5)	22 (50.0)	9 (20.5)	0.369
Absence (51)	12 (23.5)	22 (43.1)	17 (33.3)	
Vein invasion				
Presence (25)	5 (20.0)	12 (48.0)	8 (32.0)	0.670
Absence (70)	20 (28.6)	32 (45.7)	18 25.7)	

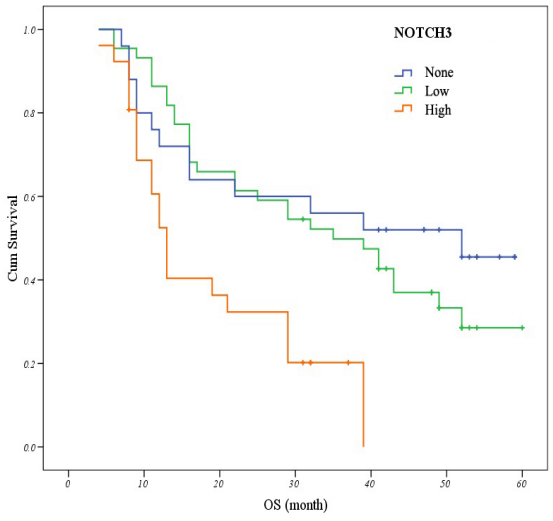

Figure 3. Kaplan-Meier survival curves based on expression of Notch3. Subgroups with high Notch3 expression had a significantly shorter survival time, compared to those with no or low Notch3 expression ($p = 0.002$, Log-Rank test).

clinical and pathological variables together with Notch3 expression status were included in a multivariate Cox regression model. Our data demonstrated that high Notch3 expression emerged as an independent prognostic factor for HCC patients (Table 2).

4. Discussion

Aberrant activation of the Notch pathway has been demonstrated in the context of many cancers, and regarded as a novel potential therapeutic target for cancers (*11*). High Notch signaling pathway activity is caused by the deregulation of a list of pathway members at different levels (such as DNA, mRNA, protein). In this study, using a meta-analysis on gene expression microarray datasets, we identified that Notch3 ranked at the top of most up-regulated Notch pathway genes in HCC at the mRNA level. Consistent with our finding, in a previous study, Gramantieri et al. also reported an upregulation of Notch3 mRNA in 95% of HCCs *vs*. normal liver tissue by *in situ* hybridization (*5*). Notch3 and its ligand Jagged1 play a key role as Notch pathway members in the pathogenesis of multiple malignancies (*12*). It has been demonstrated that Notch3 signaling affects apoptosis and tumor growth in cancers by co-operating with the EGFR-MAPK and CSL (CBF-1/RBP-Jκ, Su(H) Lag-1) pathway (*13,14*). Therefore, these data promoted the assumption that Notch3 overexpression might partially account for the increased Notch pathway activation in HCC.

Next, by an immunohistochemistry method, we obeserved a high frequency (73.7%) of Notch3 expression in HCC, similiar to the findings of Gramantieri's study in which Notch3 was not expressed in normal liver tissue, and displayed abnormal accumulation in 78% of HCC tissue (*5*). However, Gramantieri's and our data were not consistent with those of Gao *et al*.'s previous study in which cytoplasmic Notch3 expression showed no difference between HCC and adjacent non-cancerous liver samples (*6*).

In this study, we further extended the previous findings to identify a positive association between Notch3 and more aggressive phenotypes, and emphasized an independent predictor for poor prognosis in HCC. Several *in vitro* studies have revealed the relationship between Notch3 and maligant biological behavior in HCC, and the exact molecular mechanisms have also been explored. For example, Giovannini *et al*. (*15*) detected a high expression of the Notch3 intracellular domain in HepG2 cells, which suggests that Notch3 might be constitutively activated in human HCC. In another study, they demonstrated that down-regulation of Notch3 resulted in up-regulation of CDKN1C/P57, a cyclin-dependent kinase inhibitor in two HCC cell lines, accompanied by reduced cell growth (16). Giovannini *et al*. (*17*) indicated that Notch3 silencing enhances doxorubicin's chemotherapeutic effect by a p53-dependent mechanism in HCC cells, and could provide a novel strategy for HCC treatment. Together with these findings, we proposed that Notch3 plays an important regultory role in the progression of

Table 2. Univariate and multivariate analysis for prognosis in HCC

Variables	Univariate survival analyses		Multivariate survival analysis	
	HR (95% CI)*	*p* Value	Adjusted HR (95% CI)	*p* Value
Age	0.982 (0.954-1.012)	0.240	0.853 (0.388-1.875)	0.693
Gender	0.684 (0.326-1.437)	0.316	0.988 (0.956-1.021)	0.465
Tumor size	2.025 (1.169-3.509)	0.012	0.962 (0.350-2.642)	0.940
Histological grade	0.769 (0.506-1.170)	0.220	0.864 (0.516-1.447)	0.579
TNM stage	1.530 (1.177-1.988)	0.001	1.397 (1.034-1.887)	0.030
Cirrhosis	1.110 (0.657-1.878)	0.696	1.065 (0.576-1.970)	0.840
Tumor nodules	2.697 (1.449-5.020)	0.002	1.491 (0.479-4.641)	0.491
Hepatitis	1.224 (0.713-2.100)	0.463	1.314 (0.746-2.314)	0.345
Capsular formation	1.289 (0.781-2.127)	0.321	1.031 (0.584-1.821)	0.916
Vein invasion	0.575 (0.337-0.981)	0.042	0.683 (0.382-1.221)	0.198
Notch3	1.858 (1.264-2.731)	0.002	1.578(1.036-2.402)	0.034

*: HR, Hazards ratios; CI: confidence interval.

HCC, and could be regarded as a candidate for potential therapeutic targets for this maligancy.

In conclusion, in this study, we identified Notch3 as ranked at the top of overexpressed Notch pathway-related genes on datasets from the Oncomine database. We validated an aberrantly increased Notch3 expression in HCC, and found an association between its expression with more progressive traits and short survival in HCC. In addition, we also confirmed Notch3 to be an independent prognostic factor for HCC. Such knowledge should also provide novel ideas to develop better targeted therapeutic strategies that could significantly enhance HCC treatment and patient survival in the future.

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